Description

The present invention relates to a new process of proportioning of the carnitine, and more particularly an electrochemical process for the proportioning of L-carnitine, it is & statement of the butyric acid L-hydroxy-3 trimééthylamino-4, (Ch3)35-ch2chch2co. It also includes/understands one say
Positive OH for the realization of this process, in particular a particular electrode for the measurement of the currents proportional to the concentration in carnitine in a given solution, when a potential difference is applied to this one.

The determination of the concentration in Lcarnitine in various mediums, is of practical interest due so that this compound plays an essential role in the oxidation of the fatty acids in the animal organization; deficiency in L-carnitine is likely to give place to syndromes which one can cure if one connait the content of this substance. L-carnitine was also proposed like agent of investigation thyroldien. This substance is rather widespread in nature: one finds it in the animal fabric majority, in particular in the muscle, the plants and yeasts.

The method of proportioning of L-carnitine, used until now, consists of enzymatic acetylation by means of acéétyl-coenzyme A, which leads to the release of coenzyme CoA-HS in quantity equivalent to that of the carnitine brought into play. CoA-HS is then proportioned by one of the known methods. One finds of it a description detailed of PEARSON, TUBBS and CHASE in "Methods of
Enzymatic Analyses ", vol. 4, p.1758 (Editor BERGMEYER
Chem. Academic Press, New York 1976).

Coenzyme A, formed in the reaction
Acéétyl-CoA + L-carnitine carnitine

acéétyltransféérase Acéétylcarnitine+CoA-HS is proportioned by one of the following ways.

By the thiokinase
CoASH+ATP+sorbate

Sorbyl CoA + AMP + PPi
Thiokinase
One measures the increase in absorptance with 300 Nm corresponding to the sorbyl CoA.

By the oxo-2 glutarate déshydrogéénase:
CoASH + 2-oxoglutarate+NAD – oC02 + succinyl CoA+NADH+H+
One measures the increase in absorptance with 340 Nm corresponding to the NADH. These two methods require highly purified enzymes and are of a rather complicated employment.

By chemical way, using the DTNB (acid-dithio-5,5 (a) p-nitro-2 benzoTque) which reacts with CoASH to give the anion thio-5 nitro-2 benzoate which strongly absorbs with 412 nmi this method is limited by the fact that certain biological extracts contain substances which reduce the DTNB.

Ultimately, whatever the alternative of the traditional method, one encounters difficulties, of which largest east need for having a purified medium, free from substances which distort the results of the analysis..

The present invention brings an improvement sensitive to the state of this technique by the realization of a process of proportioning of the carnitine, which allows obtaining very precise results, without requiring the purification of the sample to be analyzed. The process following the invention makes possible the specific and exact determination of the content of carnitine in various biological environments, being able to contain various impurities, in particular proteins.

The new process is characterized in that one makes act of the nicotinamide adéénine dinuclééotide in his oxidized form, NAD+, on the carnitine of the medium studied, in the presence of the enzyme, carnitine dééshydrogéénase, in order to transform the carnitine into dééhydrocarnitine, with the costs of the NAD which passes to I' state of its reduced form NADH. The latter can be proportioned ampééroméétriquement, by a known method in oneself, and to provide a size equivalent to that of the carnitine thus oxidized.

The basic reaction of the invention can be written:
carnitine
L-carnitine +NAD+

dééhydro-3 carnitine +NADH+H
The formed NADH can be determined, for example, by the method of THOMAS and CHRISTIAN "Amperometric measurement of hexacyanoferrate (III) coupled dehydrogenase reactions" (Analyt.Chim.Acta, 82,265 - 1976). In this method NADH is rééoxydéé in NAD by means of the ion ferricyanide with, like enzyme, of the diaphorase, following
NADH + 2[Fe(CN)]

NAD+ + 2 èè(CN) + H+
The operations according to the invention can be carried out at various temperatures, in particular between 0 and 400CI and preferably between 150 and 300C.

In a mode of realization of the invention, one uses a sensor consisted an electrode of turntable covered with a permeable semi membrane which delimits with metal a reaction vessel, of a few tens of microns thickness, containing a mixture of carnitine dééshydrogéénase, of diaphorase, NAD and of ferricyanide.

When this sensor is put in contact with a solution of Lcarnitine, the substrate diffuse through the membrane, the carnitine dééshydrogéénase catalyses the oxidation of L-carnitine by the NAD+, the diaphorase rééoxyde the NADH with the costs of ferricyanide, itself regenerated electrochemically with the electrode according to the following diagram

(1): carnitine dééshydrogenase (Z): diaphorase the intensity of courrant crossing the circuit is proportional to the concentration in L-carnitine.

Although the ion ferricyanide is appropriate particularly for the proportioning of the formed NADH. this proportioning can be also carried out by means of other reagents able to make pass NADH to the state of NAD; such are for example oxygen, some dyes, the cytochrome, etc.

The device following the invention - in its broad outline - is built like those of the former technique. An english bond, according to fig. 1, includes/understands a tube or stem L closed in bottom by a semipermeable membrane 2, fixed at the wall external of the tube by means of a ring 3. Above the membrane is a disc 4 out of conducting matter 4, stainless, connected by a driver 5 on the terminal + of a source of continuous tension. Between disc 4, forming the anode, and membrane 2 remains a small space 6 which constitutes a reaction vessel; it is in this room that one places the solution of enzyme to be used. The discussion thread 7 constitutes the cathode.L' unit L to 7, which forms the enzymatic sensor, plunges in a container 9 container the reagents necessary to proportioning and the solution studied 8. The sensor is assembled in series with a source of tension continues E and a resistance R, as schematically shown on figure 3. Catches of recording are at the boundaries of resistance R.

Here as an example nonrestrictive a mode of realization of the invention.

Electrode 4 (figure 1) is consisted a platinum disc 3 mms in diameter, enchasséé in a cylindrical tube L out of plastic, covered with a semipermeable membrane 2 in cellophane, whose threshold of cut is approximately 12000. The reaction vessel 6, delimited by the electrode 4 and membrane 2, has a volume of úúl and contains the two enzymes.

The speed of the electronic transfer Fe(CN)6 = Fe(CN)6 + E on Pt electrode makes possible the use of a device to two electrodes slightly polarized. The auxiliary electrode is a Pt electrode. A potentiostat makes it possible to impose a potential 0,3 V between cathode and the anode. The intensity of the current of electrolysis is measured using a resistance of 100 Kr. placed in series in the circuit and, at the boundaries of which a recorder of strong impedance is connected. A voltmeter with high impedance of entry in addition makes it possible to control the potential difference between the two electrodes during electrolysis.

The enzymatic solution of the reaction vessel is consisted 3 SSL of a solution of plug phosphates 50 m M pH 7,5, containing 2 units of carnitine déshydrogénase of Pseudomonas putida (EC.1.1.1. 108) and 5 units of diaphorase of micro-organism (EC. 1.6.99.). The electrolytic solution of 5 ml is a plug TRIS/HCl 50 mm pH 9, 16 mm out of potassium ferricyanide and 10 mm in NAD+.

The experimental conditions were selected so that the limiting stage of the process is the speed of diffusion of L-carnitine through the membrane. In the stationary state the intensity of the current is then directly proportional to the concentration of
L-carnitine in the external solution to proportion.

Measurement is carried out in the following way. The electrode is plunged in the electrolytic solution which is agitated using a magnetic stirrer, and one awaits the stabilization of the basic current. Then one adds L-carnitine: this one diffuses through the membrane and is oxidized by the carnitine déshydrogénase. One records the curve giving the intensity of the current according to time. At the end of approximately two minutes, one obtains a stage corresponding at the stationary state (fig. 2). One Re comes to the base line into 10 to 15 minutes/rlnççant ltélec- trode in the solution from electrolyte which is used for following measurement.

These operations are illustrated by the graphics of the fig. 2, of I = f(t): A indicates the moment of injection of L-carnitine, B the stage of the current and C - D the return to the base line (rinsing of the sensor).

The layout of the curve of the intensity of the current in a stationary state, function of the concentration in L-carnitine, makes it possible to obtain a line for concentrations in L-carnitine ranging between 0,1 and 2 mm, which corresponds to currents ranging between 0,05 jiA and 1,1 A. the precision of measurements is very 2% in impure medium, therefore comparable with enzymatic proportionings using the acétylcarnitine transférase in suitably purified medium.

The invention is illustrated for the nonrestrictive examples which follow.

EXAMPLE 1
A standard calibration of the electrode with L-carnitine is obtained in the following way.

The electrode is plunged in 5 ml of electrolytic solution agitated using a bar magnet. After stabilization of the basic current, one injects 10 Al of a solution to 50 mm in L-carnitine. At the end of two minutes the current crossing the system reaches the stage of 0,05 ssA. The electrode is then soaked in 5 ml of electrolytic solution for rinsing, the return to the base line is done at the end of 8 minutes (Cd fig. 2).

One repeats measurement with 20, 50, 100, 150 and 200 L of the solution 50 m M in L-carnitine.

The recorded currents are respectively 0,1 A 0,28 ; 0,55 LLA ; 0,8 A et 1,1 ssA. The layout of the curve Intensity (A A) according to the concentration in Lcarnitine is a line.

EXAMPLE 2

a study of the ageing of the electrode was carried out during 10 days. Each day one plots a calibration curve as described in example 1. Between two series of measurements, the sensor is preserved at 40C in a solution of plug phosphates 50 mm pH 7,5. The response of the sensor remains linear during the 10 days for concentrations in L-carnitine ranging between 0,1 and 2 mm, but the intensity of the recorded current decreases each day, to reach the 10th day 15% of that of the beginning.

EXAMPLE 3 the electrode with L-carnitine made it possible to follow in 70 hours the advance of a reaction of bioconversion of the déehydro-3 carnitine in L-carnitine, catalysed by the carnitine déeshydrogéénase. One used an engine of 2 liters equipped with an adjusted thermostat with 300C; it contained 1 liter of an aqueous medium of the following components phosphates sodium and of potassium 50 mm, pH 7,5 NAD+, 0,6 mm carnitine déeshydrogéénase 800 units formate déeshydrogéénase 500 units formate of sodium 150 mn chloramphenicol 120 Mg. One injected a solution 0,8 then hydrochlorate molar of déeshydro-3 carnitine and 0,8 molar in formic acid. The speed of injection was of 1,2 ml/h. The pH was maintained to 7,5 per addition of ammonia 2N controlled using a pH-meter with automatic titration. Every two hours one takes an aliquot portion of the solution of bioconversion suitably diluted in plug phosphates pH 7,5 50 mm, in order to have a concentration in L-carnitine in the electrolytic solution close to 1 mm Ainsi proportions one the carnitine throughout the bioconversion.

EXAMPLE 4 the sensor with L-carnitine made it possible to control the effluents of an exchanging chromatography of ions of tinéée to purify L-carnitine obtained at the end of the reaction of bioconversion. 290 ml of a solution 364 mm in L-carnitine (17g) are charged in a column with 300 ml of cation resin IR 120. The column is rinsed with water (1 liter) and the content of L-carnitine ae the effluent is controlled using the sensor; that made it possible to note that all the carnitine deposited does not remain not hung on the resin. Onréécupèère thus, in water of washing, 9 G of L-carnitine not retained by the column. L-carnitine remaining (8 G) is ééluéée with an ammonia solution 2 N (500ml).

EXAMPLE 5 to limit consumption in NAD, one used a NAD grafted on a water-soluble polymer, Polyééthylèèneglycol 20000, which is imprisoned in the reaction vessel with the two enzymes. The response of the sensor is always linear for concentrations in L-carnitine between 0,1 mm and 2 mm but the sensitivity are decreased by 2. A similar test was carried out with dextrane 25000 as a polymer support; the same results were obtained.

Claims

1. Process of proportioning of the carnitine by enzymatic way, characterized in that one makes act of the nicotinamide adéénine dinuclééotide in his oxidized form, NAD+, on the carnitine of the medium studied, in the presence of carnitine dééshydrogéénase, in order to transform the carnitine into dééhydrocarnitine, and which one ampééroméétriquement proportions reduced form NADH resulting.

2. Proceeded according to claim 1, characterized in that the NADH is proportioned enzymatiquement by oxidation by means of the ion ferricyanide, in the presence of diaphorase.

3. Proceeded according to claim 1, characterized in that the NADH is proportioned by oxidation by means of oxygen.

4. Proceeded according to claim 1, characterized in that the NADH is proportioned by oxidation by means of a dye.

5. Proceeded according to claim 1, carac terized in what the NADH is proportioned by oxidation by means of cytochrome.

6. Device for the realization of the process following one of the claims 1 to 5, which includes/understands a formed sensor of a tube or a stem (1) whose lower end carries a semipermeable membrane (2) vis-a-vis an electrode (4), a reaction vessel (6) very low depth being envisaged between the membrane (2) and the electrode (4), characterized in that this room contains an aqueous solution of carnitine dééshydrogéénase, in a plug of pH.

7. Device following claim 6, carac terized in what the solution contained in the room (6) also contains an enzyme able to promote the reoxidation of the NADH in NAD+ by a reagent of oxidation, in particular the diaphorase.